Auxin Regulation of the Gibberellin Pathway in Pea[1]

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The auxin indole-3-acetic acid (IAA) has been shown to promote the biosynthesis of the active gibberellin (GA_1) in shoots of pea (*Pisum sativum*). We used northern analysis to investigate the timing of IAA-induced changes in transcript levels of *PsGA3ox1* (Mendel's *LE*), *PsGA2ox1*, *PsGA2ox2*, and *PsGA20ox1*, key genes for the later stages of GA_1 biosynthesis and metabolism in pea. Rapid (2–4 h) changes occurred in the transcript levels of *PsGA3ox1*, *PsGA2ox1*, and *PsGA2ox2* after treatent with IAA. [^{14}C]GA_1 metabolism studies in decapitated shoots indicated that IAA inhibits GA_1 deactivation, consistent with the suppression of *PsGA2ox1* (*SLN*) transcript levels by IAA. Studies with the *sln* mutant also indicated that *PsGA2ox1* activity is involved in GA_1 deactivation in decapitated shoots. Culture of excised internode tissue in the presence of auxin clearly demonstrated that internode tissue is a site of GA_1 biosynthesis per se. Excised internode tissue cultured in the presence/absence of cycloheximide showed that de novo protein synthesis is required for IAA-induced increases in *PsGA3ox1* transcript levels. Auxin dose response studies indicated that IAA concentration is a critical determinant of GA_1 biosynthesis over 1 to 2 orders of magnitude, and a range of auxins was shown to affect the GA pathway.

Recent studies have highlighted the importance of hormone interactions or "cross talk," and how these interactions can affect both hormone biosynthesis and signal transduction (Kende et al., 1998; Grossmann, 2000; Ross and O'Neill, 2001; Peeters et al., 2002; Swarup et al., 2002). In a previous paper (Ross et al., 2000), we demonstrated a new link between the "classical" plant hormones auxin and gibberellin (GA). In wild-type (WT) pea (*Pisum sativum*) plants, it was shown that decapitation (removal of the apical bud) dramatically reduced both the level of endogenous GA_1 (the bioactive GA) in expanding internodes and the recovery of the [$^{13}C^3H$]GA_1 peak from internodes fed [$^{13}C^3H$]GA_{20}. Consistent with those findings, decapitation reduced the transcript level of Mendel's *LE* gene, which encodes a GA 3-oxidase (PsGA3ox1) for the conversion of GA_{20} to GA_1 in pea shoots, and increased transcript levels of the *SLN* gene, which encodes a GA 2-oxidase (PsGA2ox1) for the conversion of shoot GA_{20} to the inactive GA_{29} (Ross et al., 2000; Fig. 1). Addition of the auxin indole-3-acetic acid (IAA) to the "stump" of decapitated plants completely reversed these effects, restoring the [$^{13}C^3H$]GA_1 peak in internodes fed [$^{13}C^3H$]GA_{20} to at least the level found in intact plants, whereas the endogenous level of GA_1 after IAA application was vastly increased compared with control decapitated plants and was three times the level in intact plants (Ross et al., 2000). Supporting this finding was the strong up-regulation in *PsGA3ox1* transcript levels and down-regulation of

PsGA2ox1 transcript levels after IAA application (Ross et al., 2000).

However, the above results leave unanswered key questions regarding the nature of the IAA/GA interaction. These results were obtained 2 d after decapitation (Ross et al., 2000) and provide little information on how rapidly IAA affects the GA pathway. The changes observed might have been simply secondary, long-term consequences of auxin action rather than changes that potentially mediate some of the effects of IAA on elongation. Another important question is: Does IAA affect other GA genes, apart from *PsGA3ox1* and *PsGA2ox1*? Furthermore, does IAA affect GA_1 deactivation? We have previously shown that the gene *PsGA2ox1* (*SLN*) is down-regulated by IAA, but the role of this gene in GA_1 deactivation in vivo is uncertain (Ross et al., 1995; Lester et al., 1999). Here, we also investigate whether IAA promotes GA_1 biosynthesis in the internodes themselves: Our previous data do not preclude the possibility that IAA acted in leaves to stimulate the synthesis of GA_1 that subsequently moved into the internodes. In addition, we examine whether *LE* (*PsGA3ox1*) is a "primary" auxin response gene. Recent studies have uncovered a suite of genes whose expression is rapidly and specifically altered by auxin (for review, see Guilfoyle, 1998). These primary or "early" auxin response genes respond to auxin at the transcript level, in the absence of de novo protein synthesis. We also investigate the quantitative nature of the IAA-GA interaction. Is a threshold level of IAA necessary for GA_1 biosynthesis, above which GA_1 synthesis is normal, and below which it is not produced? Or is there a linear relationship between the two hormones? Finally, do other auxins, including well-known herbicides, also affect the GA pathway?

In this study, we monitor the effects of IAA application on *PsGA3ox1*, *PsGA2ox1*, *PsGA2ox2*, and
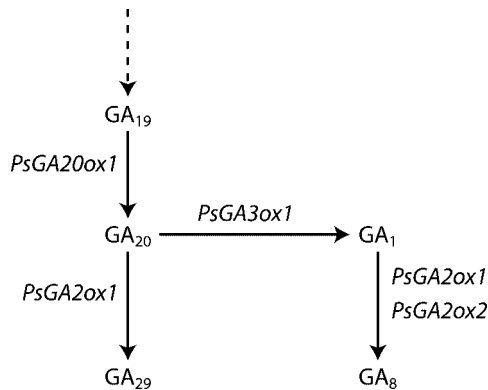
Figure 1. Later stages of the GA pathway in pea shoots. Sites of action of major genes are shown. Both *PsGA2ox1* and *PsGA2ox2* have been shown to convert GA_1 to GA_8 in vitro (Lester et al., 1999).

PsGA20ox1 transcript levels (Fig. 1) over an 8-h time course to determine how rapidly IAA affects GA gene expression and whether IAA affects the latter two genes. We also examine the effects of IAA on the metabolism of exogenously applied $[^{14}C]GA_1$, and we use the *sln* mutation to further examine the role of *PsGA2ox1* in GA_1 deactivation. In addition, we report on GA_1 biosynthesis in isolated stem segments cultured on liquid Murashige and Skoog (1962) medium with and without IAA. This system is then used to study the effects of the protein synthesis inhibitor cycloheximide (CHX) on *PsGA3ox1* expression to investigate whether *PsGA3ox1* is an early or a late auxin response gene. The effects of IAA concentration on endogenous GA_1 levels and internode elongation are also examined using a range of IAA concentrations in lanolin applied to the stump of decapitated plants. We also examine the effects of other naturally occurring and synthetic auxins on the GA pathway.

RESULTS

Rapidity of the IAA Response

IAA-induced up-regulation of *PsGA3ox1* transcript levels was apparent 2 h after the application of the hormone (Fig. 2A, compare lanes 3 and 4). The *PsGA3ox1* transcript level continued to increase in IAA-treated samples until 8 h after application, the last time point in this experiment (Fig. 2A). The *PsGA3ox1* transcript level in intact samples was comparable with that in decapitated peas 2 h after IAA application (Fig. 2A, compare lanes 11 and 4).

In contrast, IAA application down-regulated the level of *PsGA2ox1* transcript, and this effect was clear 4 h after IAA application (Fig. 2B, compare lanes 5 and 6). *PsGA2ox1* transcript levels declined further in IAA-treated plants by the 8-h time point (Fig. 2B, compare lanes 9 and 10).

The effect of IAA application on the transcript level of *PsGA2ox2* (Fig. 2C) was in complete contrast to the

effects on *PsGA2ox1*. Four hours after IAA application, a strong increase in *PsGA2ox2* transcript was observed in comparison with control decapitated plants (Fig. 2C, compare lanes 5 and 6). The level of *PsGA2ox2* transcript in IAA-treated plants increased further up to the final time point (Fig. 2C, lanes 6, 8, and 10), whereas the level of transcript in decapitated controls remained low (Fig. 2C, lanes 5, 7, and 9). It is interesting to note the low level of *PsGA2ox2* transcript in intact, untreated plants (Fig. 2C, lane 11).

IAA had little or no effect on the transcript level of the shoot 20-oxidase gene *PsGA20ox1* (Fig. 2D). This
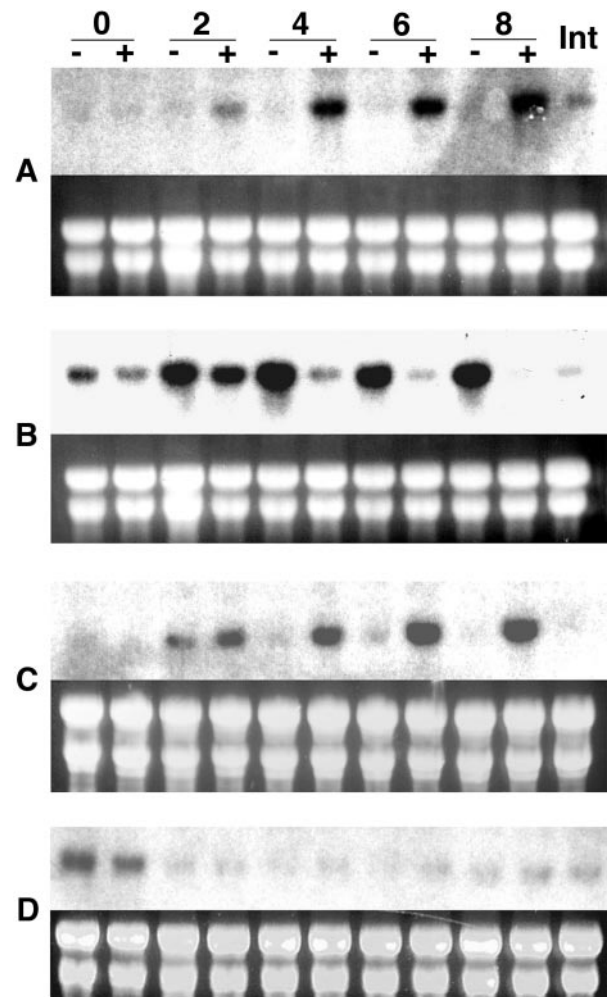

Figure 2. Short-term effects of IAA on GA gene expression. Shown are *PsGA3ox1* (A), *PsGA2ox1* (B), *PsGA2ox2* (C), and *PsGA20ox1* (D) transcript levels in the uppermost elongating internode of decapitated peas treated with IAA/lanolin (+) or lanolin only (−). Internodes were harvested 0, 2, 4, 6, and 8 h after application. All plants (except intact controls) were decapitated for 36 h before the commencement of the time course study. IAA/lanolin or lanolin was reapplied 4 h after the initial application. Intact peas (Int) were harvested at the same time as 8-h samples. Corresponding gels showing ribosomal RNAs stained with ethidium bromide are shown immediately below each northern blot, to indicate the loading of lanes. Exposure times were as follows: *PsGA3ox1*, 48 h; *PsGA2ox1*, 72 h; *PsGA2ox2*, 24 h; and *PsGA20ox1*, 48 h.

result is supported by a recent study, which shows that IAA has no effect on *PsGA20ox1* transcription in pea pericarp (Ngo et al., 2002). Also, there were only subtle differences in the level of endogenous GA_{19} and GA_{20} in intact, decapitated, and decapitated IAA-treated peas (Ross et al., 2000), suggesting that the conversion of GA_{19} to GA_{20} (catalyzed by the *PsGA20ox1* gene product) is not regulated by IAA.

It is worth noting that the uppermost 5 to 10 mm of harvested tissue in the above time-course experiments was removed before RNA extraction took place to avoid contamination of the sample with IAA/lanolin. Because IAA has been shown to move in a basipetal fashion at a rate of 5 to 20 mm h^{-1} (Lomax et al., 1995), it would be reasonable to suggest that even at 2 h after application, sections of the internodes harvested (which exceeded 20 mm in length), might not yet have received the IAA applied. Hence our data would, if anything, underestimate the rapidity of the response to IAA.

Overall, it appears that IAA promotes the transcription of both *PsGA3ox1* and *PsGA2ox2* and inhibits the transcription of *PsGA2ox1* in a rapid (2–4 h) and sustained manner. The inclusion of controls at each time point revealed underlying variation of transcript levels with time in the early stages of the time course. For example, the transcript level of *PsGA2ox2* was increased and the level of *PsGA20ox1* reduced in both control and IAA-treated plants 2 h after the commencement of the experiment (Fig. 2, C and D). Also, the level of *PsGA2ox1* transcript in control plants increased at both 2- and 4-h time points (Fig. 2B). Garcia-Martinez and Gil (2002) also found variation with time in the mRNA level of a key GA gene (*PsGA20ox1*) and noted that the physiological significance of this variation is presently unclear.

The Effect of IAA on GA_1 Deactivation

Northern analysis showed that IAA application down-regulates *PsGA2ox1* and up-regulates *PsGA2ox2* transcript levels (Fig. 2, B and C). Because the *PsGA2ox2* gene product is the favored candidate for the deactivation of GA_1 to GA_8 in pea shoots (Lester et al., 1999), it was vital to examine the effect of IAA application on the metabolism of GA_1 to further understand the gene(s) responsible for this process. To this end, $[^{14}C]GA_1$ was applied to decapitated plants treated with pure lanolin or with lanolin containing IAA (3,000 μg g^{-1}). In control plants, 61.7% ± 1.6% ($n = 2$) of radioactivity extracted from internode 8-9 with GA_8, and only 23.0% ± 1.1% with co-eluted unmetabolized GA_1 (Fig. 3A). In contrast, in IAA-treated internodes, only 19.9% ± 1.0% ($n = 2$) of recovered radioactivity co-eluted with GA_8 and 70.9% ± 1.2% with GA_1 (Fig. 3B). These data indicate that increasing the IAA content of the internodes strongly inhibited the conversion of GA_1 to GA_8.
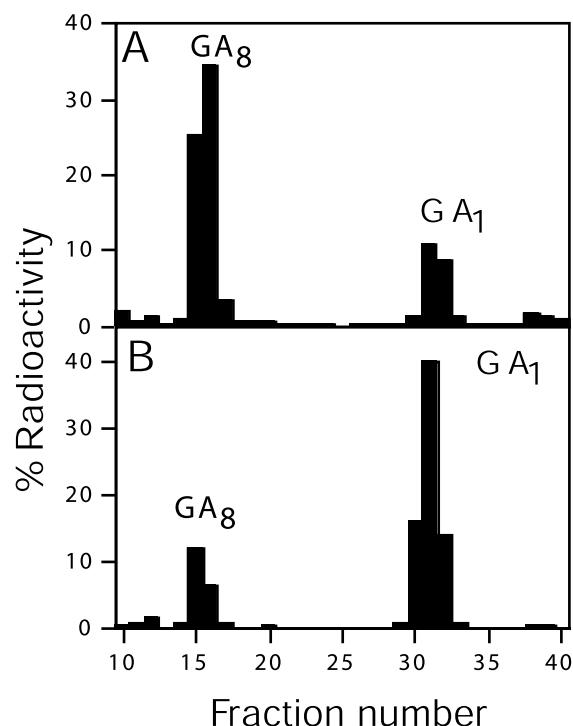

Figure 3. Effects of IAA application on the metabolism of $[^{14}C]GA_1$ in decapitated peas. Shown is the metabolism of $[^{14}C]GA_1$ to $[^{14}C]GA_8$ in decapitated peas (A) and decapitated peas treated with IAA (B; 3,000 μg g^{-1}). $[^{14}C]GA_1$ was applied to leaf 8, and internode 8-9 tissue was harvested 10 h after application of the substrate. $[^{14}C]GAs$ were chromatographed as methyl esters. Identities of peaks are indicated. Data are shown as the percentage of total radioactivity in the HPLC run. The levels of radioactivity recovered were: controls, 19.5 ± 1.8 dpm × 10^{-3}; IAA treated, 31.9 ± 3.8 dpm × 10^{-3}; $n = 2$.

GA_1 Deactivation in the *sln* Mutant

Given that IAA application both up-regulates *PsGA2ox2* transcript levels and inhibits the deactivation of GA_1, it is doubtful that the *PsGA2ox2* gene product is primarily responsible for GA_1 deactivation in decapitated pea shoots. Therefore, we examined the role of *PsGA2ox1* in GA_1 deactivation, using the *sln* mutation (Lester et al., 1999). The *sln* mutation results in a premature stop codon because of a single-base deletion, which leads to the formation of a truncated, nonfunctional PsGA2ox1 protein (Lester et al., 1999). By comparing GA_1 metabolism in decapitated *sln* and WT peas, we could examine GA_1 deactivation in the absence of *PsGA2ox1* activity. It is clear that the loss of *PsGA2ox1* gene activity in the *sln* mutant severely reduced the recovery of $[^{14}C]GA_8$ after application of $[^{14}C]GA_1$ when compared with WT plants (Fig. 4). In *sln* plants, 72.1% ± 2.7% of total radioactivity extracted from internode 9-10 co-eluted with unmetabolized GA_1 and only 7.5% ± 0.6% with GA_8 (Fig. 4A, $n = 2$). In contrast, internode 9-10 tissue from WT plants contained 38.6% ± 0.02% of total radioactivity co-eluting with GA_1 and 38.0% ± 1.9% with GA_8 (Fig. 4B, $n = 2$). These results suggest that *PsGA2ox1* activity is important for the deactivation of
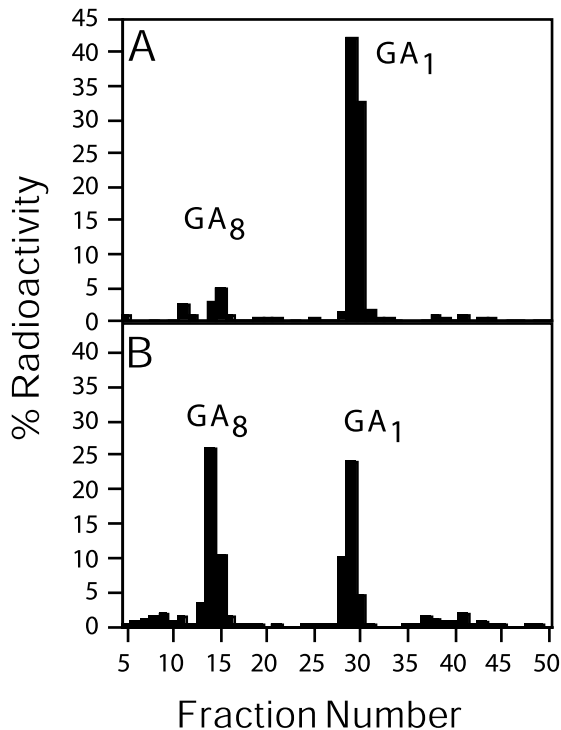
Figure 4. Effects of the *sln* mutation on [^{14}C]GA$_1$ metabolism in decapitated peas. Shown is the [^{14}C]GA$_1$ metabolism in decapitated *sln* (A) and decapitated *SLN* (B) peas. Plants were decapitated above node 10, left for 19 h, and then treated with [^{14}C]GA$_1$ at a rate of 80,000 dpm plant^{-1} on leaf 9 in 10 μL of ethanol. Five hours after application of [^{14}C]GA$_1$, the treated leaf was excised, and internode 9-10 was harvested 9 h after application. [^{14}C]GAs were chromatographed as methyl esters. Data are shown as the percentage of total radioactivity in the HPLC run. Identities of peaks are indicated.

GA$_1$ to GA$_8$ in decapitated pea shoots, whereas *PsGA2ox2* activity plays a minor role. It remains possible, however, that *PsGA2ox2* is the main GA$_1$ deactivation gene in intact plants (Lester et al., 1999).

GA$_{20}$ Metabolism in Excised Stem Segments

We used excised pea stem segments floated on liquid Murashige and Skoog medium as a system to investigate the effects of IAA on GA$_1$ biosynthesis in the absence of root, leaf, and apical tissue. This work expands on the study of Ross et al. (2000) using decapitated peas by further isolating the expanding internode. The system has the advantage of allowing the addition and quick uptake of IAA and other compounds as well as radiolabeled GA substrates for use in GA metabolism studies.

Sherriff et al. (1994) reported no evidence of GA$_1$- or GA$_8$-like peaks when excised *LE* stem segments, cultured in liquid Murashige and Skoog, were fed [^{13}C^{3}H]GA$_{20}$. Earlier, Smith (1992) proposed that GA$_1$ was a product of GA$_{20}$ in Murashige and Skoog-cultured stem tissue, but Sherriff et al. (1994) suggested that the product concerned was in fact GA$_{29}$-catabolite. The subsequent work of Ross et al. (2000)

suggests that the lack of GA$_1$-like peaks (and therefore a lack of GA$_8$-like peaks) reported by Sherriff et al. might be attributable to the absence of IAA in the culture medium. In the current study, [^{14}C]GA$_{20}$ was added to the liquid Murashige and Skoog without IAA, and the results supported the findings of Sherriff et al. (1994): After 6 h, the main metabolite recovered from the segments was [^{14}C]GA$_{29}$ (Fig. 5A). In contrast, when the medium contained 5 mg L^{-1} IAA, the major metabolites were (in decreasing order of abundance) [^{14}C]GA$_1$, [^{14}C]GA$_8$, and [^{14}C]GA$_{29}$ (Fig. 5B). The identities of metabolites were confirmed by GC-MS or by GC-MS-selected ion monitoring. After 24 h of incubation without IAA, the major metabolite was again [^{14}C]GA$_{29}$ (Fig. 5C), with more [^{14}C]GA$_{20}$ metabolized than at 6 h. After 24 h of incubation with IAA, the major metabolites were (in decreasing order of abundance) [^{14}C]GA$_8$, [^{14}C]GA$_{29}$, and [^{14}C]GA$_1$ (Fig. 5D). It is possible that in the 24-h case, GA$_1$2-oxidation was stronger than at 6 h, resulting in a relatively small GA$_1$ peak and a large GA$_8$ peak (Fig. 5D). Auxin tended to increase the recovery of unmetabolized [^{14}C]GA$_{20}$ (Fig. 5), probably by inhibiting the step [^{14}C]GA$_{20}$ to [^{14}C]GA$_{29}$.

The significance of the present findings is 2-fold. For the first time, to our knowledge, we have shown that expanding internodes of pea exhibit 3-oxidase activity per se and that a source of IAA is required for this activity to be maintained.
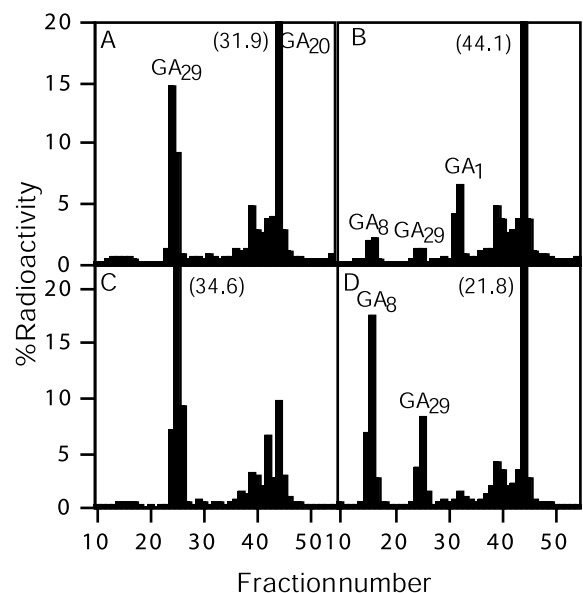

Figure 5. [^{14}C]GA$_{20}$ metabolism in isolated pea internodes cultured on Murashige and Skoog solution with and without IAA. A, Six-hour incubation without IAA; B, 6 h with IAA; C, 24 h without IAA; D, 24 h with IAA. Data are shown as percentages of total radioactivity in the HPLC run. The retention times of authentic GA standards are indicated, as is the percentage of radioactivity for peaks where these are off scale.

Effects of CHX on *PsGA3ox1* Transcript Levels

IAA up-regulated *PsGA3ox1* mRNA levels in excised stem sections, consistent with the results of the metabolism studies above. The level of *PsGA3ox1* transcript in excised segments incubated with 5 mg L^{-1} IAA was far greater than in control segments after 4 h (Fig. 6, compare lanes 1 and 2). However, in contrast to known primary auxin-responsive genes in pea (Abel and Theologis, 1996), the addition of the protein synthesis inhibitor, CHX, repressed IAA-induced *PsGA3ox1* mRNA accumulation in the excised segments (Fig. 6, compare lanes 2 and 4). These results indicate that IAA-induced up-regulation of *PsGA3ox1* transcript level requires de novo protein synthesis. To show that the reduction of *PsGA3ox1* transcript in the presence of CHX was not attributable to a severe reduction in IAA uptake from the Murashige and Skoog medium caused by CHX, the level of IAA was quantified in the harvested segments (after rinsing in distilled water). The level of IAA in segments cultured with IAA or IAA/CHX was very high: more than 10 times that found in intact peas (data not shown).

The Effect of IAA Concentration on GA_1 Levels and Elongation

To further characterize IAA-induced changes in GA_1 biosynthesis and shoot elongation, a series of lanolin pastes of increasing IAA concentration was applied to the stump of decapitated peas, and the levels of GA_1 and IAA in and the elongation of internode 6-7 were monitored. Figure 7 shows that GA_1 levels rose sharply in response to increasing IAA content, with GA_1 content leveling off at an IAA

Figure 7. Effect of IAA content on elongation and GA_1 production in decapitated peas. Shown are changes in internode elongation (□) and endogenous GA_1 level (○) of internode 6-7 tissue in response to increasing IAA. Where SE bars are not visible, they lie within the dimensions of the symbol. Points represent means of eight replicates for elongation data and two replicates for hormone levels. Elongation data represent the mean difference between the initial and final lengths of internodes.

content of around 370 ng g^{-1} fresh weight. Internode elongation also rose steadily in response to increasing IAA, but appeared to be saturated at around 150 ng g^{-1} fresh weight IAA (Fig. 7). Intact plants grown in the same experiment contained 241 ± 2.6 ng g^{-1} fresh weight IAA and 17 ± 3.1 ng g^{-1} fresh weight GA_1. This point lies on the line relating the GA_1 and IAA levels in the decapitated plants (Fig. 7), suggesting that the relationship between IAA content and GA_1 levels in decapitated plants is comparable with that in intact plants. These results also clearly illustrate, at least in a decapitated shoot system, that IAA is a crucial determinant of shoot GA_1 levels. It is interesting to note the low variation in IAA level within each treatment, because the technique of applying IAA in a lanolin paste to decapitated peas can lead to slight differences in the amount of paste applied to each decapitated stump. This suggests some mechanism by which decapitated pea shoots can regulate the uptake of exogenously applied IAA.

Effects of Other Auxins

To examine the effects of other auxins on the GA pathway, excised stem segments were cultured on liquid Murashige and Skoog containing either no auxin or the relevant auxin at a high (3×10^{-5} M) or low (3×10^{-6} M) concentration, and the metabolism of $[^{14}C]GA_{20}$ was monitored. Table I shows the percentage of total radioactivity co-eluting with GA_8, GA_{29}, and unmetabolized GA_{20} after 24 h. The percentage of $[^{14}C]GA_8$ rather than $[^{14}C]GA_1$ was used as a means of comparison, because the long culture time meant that the vast majority of $[^{14}C]GA_1$ pro-
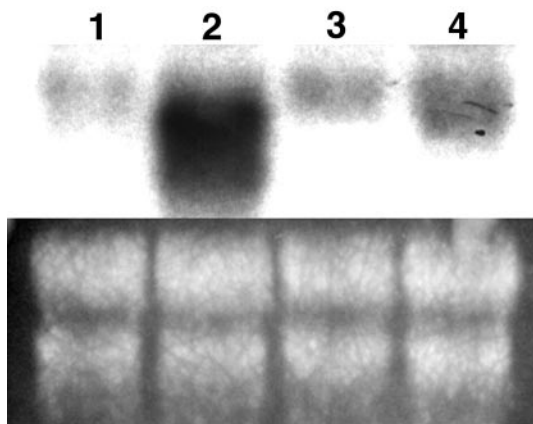
Figure 6. Effects of IAA and CHX on *PsGA3ox1* transcript levels. Shown are *PsGA3ox1* transcript levels in excised pea internodes cultured for 4 h on Murashige and Skoog medium containing the following: 1, no added IAA or CHX; 2, IAA (5 μg mL^{-1}); 3, CHX (14 μg mL^{-1}); 4, IAA (5 μg mL^{-1}) and CHX (14 μg mL^{-1}). The corresponding gel showing ribosomal RNAs stained with ethidium bromide is shown immediately below the northern blot to indicate the even loading of 5 μg of total RNA per lane.
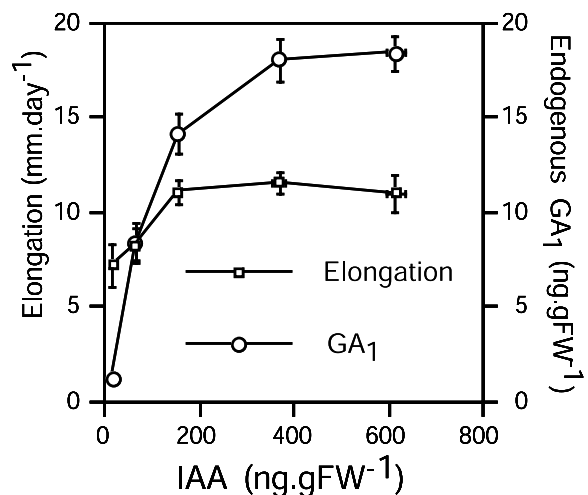
Table I. *Effects of auxin type on [^{14}C]GA$_{20}$ metabolites in excised internode 7-8 tissue cultured in sterile liquid Murashige and Skoog at 20° C for 24 h*

Auxins were present at a concentration of 3×10^{-5} M. Data are percentages of radioactivity in HPLC run co-eluting with GAs shown.

Treatment	[^{14}C]GA$_8$ (Fractions 15 and 16)	[^{14}C]GA$_{29}$ (Fractions 23 and 24)	[^{14}C]GA$_{20}$ (Fractions 43 and 44)
Control	<1	47	13
IAA	19	24	20
NAA	17	28	15
2,4-D	13	28	15
4-Chloro-IAA	13	29	16
Indole-3-butyric acid (IBA)	6	38	22

duced was converted to [^{14}C]GA$_8$ at the time of harvest. In the control case, the main metabolite of [^{14}C]GA$_{20}$ was again the inactive [^{14}C]GA$_{29}$ (47%), with only very small amounts (<1%) of GA$_8$ detected. The addition of IAA to the growth medium, as previously shown (Fig. 5B), led to the production of a significant [^{14}C]GA$_8$ peak in comparison with the [^{14}C]GA$_{29}$ peak. All other auxins tested, whether synthetic or naturally occurring, also resulted in clear [^{14}C]GA$_8$ peaks and a reduction in the [^{14}C]GA$_{29}$ peak (Table I). IBA was the least effective auxin in this regard. These results show that the promotion of GA$_1$ biosynthesis is not limited to IAA and that auxin/GA interactions may occur in tissue types where some of these other auxins are found.

DISCUSSION

This study shows clearly that IAA can regulate, in a rapid and coordinated manner, key genes encoding enzymes for GA biosynthesis and deactivation in pea internodes. IAA up-regulated expression of *PsGA3ox1* (Mendel's *LE*) after 2 h (Fig. 2), indicating that *LE* is an authentic auxin-responsive gene, rather than a gene whose expression is merely maintained indirectly via the effects of auxin on growth (Guilfoyle, 1998). IAA also rapidly down-regulated expression of the GA deactivation gene *PsGA2ox1* (*SLN*; Fig. 2). This establishes that variations in the supply of IAA from the apical bud would be expected to result in rapid changes (within hours) in gene expression and ultimately GA levels in the elongating internodes of the pea plant. Interestingly, IAA affected *PsGA2ox2* in the opposite way to *PsGA2ox1*. It is possible that *PsGA2ox2* is not directly regulated by IAA and that the apparent IAA-induced up-regulation of this gene (Fig. 2) is attributable to the feed-forward phenomenon, whereby bioactive GAs up-regulate genes encoding GA deactivation enzymes (Thomas et al., 1999; Elliott et al., 2001). According to this theory, IAA treatment would have increased GA$_1$ content, thereby up-regulating *PsGA2ox2* transcript levels (Fig. 2). Elliott et al. (2001) showed that in differing pea genotypes, high GA$_1$ content is associated with high *PsGA2ox2* (and *PsGA2ox1*) transcript levels. We suggest that in the

case of *PsGA2ox1*, applying auxin to decapitated plants overrides this feed-forward effect.

Evidence That IAA Inhibits the Step GA$_1$ to GA$_8$

The data in Figure 3 indicate that application of IAA to decapitated peas inhibited the deactivation of [^{14}C]GA$_1$ to [^{14}C]GA$_8$. This is consistent with the finding that IAA reduces *PsGA2ox1* mRNA levels (Fig. 2B). However, it had not been demonstrated previously that *PsGA2ox1* (*SLN*) actually plays a role in GA$_1$ deactivation in vivo. Although the *sln* mutation completely blocked GA$_1$ deactivation in vitro (Lester et al., 1999), the mutation did not markedly affect GA$_1$ metabolism in intact plants (Ross et al., 1995). Here, we present evidence that *SLN* does play a role in vivo: In decapitated plants, *sln* appeared to reduce the conversion of [^{14}C]GA$_1$ to [^{14}C]GA$_8$ (Fig. 4). This indicates that *PsGA2ox1* is the gene primarily responsible for the deactivation of GA$_1$ in decapitated peas. We suggest that decapitation reveals a role for *PsGA2ox1* in vivo by removing the inhibition of *PsGA2ox1* expression by IAA from the apical bud. Thus, decapitation markedly promotes GA$_1$ deactivation in WT plants but not in the *sln* mutant, where the PsGA2ox1 protein is nonfunctional.

Excised Pea Internodes Have 3-Oxidase Activity

Analysis of GA$_{20}$ metabolism and *PsGA3ox1* mRNA levels in excised stem segments of pea clearly shows that this tissue is a site of *PsGA3ox1* expression (Fig. 6) and GA$_1$ production (Fig. 5). This is the first time, to our knowledge, that isolated stem tissue of pea, or any isolated section of pea, has been unequivocally shown to exhibit *PsGA3ox1* activity. Past studies have attempted to culture excised pea stem segments to investigate the effect of the *le* mutation on the metabolism of applied radiolabeled GA$_{20}$ (Smith, 1992; Sherriff et al., 1994). In light of current results, the lack of GA$_1$ peaks observed by Sherriff et al. (1994) was attributable to the absence of auxin in the incubation medium, which resulted in the majority of applied GA$_{20}$ being converted to GA$_{29}$ (Fig. 5). The present finding is important because the elongating

internodes themselves can now be seen as a site of GA_1 biosynthesis. The promotion of GA_1 biosynthesis in isolated stem sections implies that now, even in this traditional system for studying auxin-induced growth, it cannot be discounted that IAA promotes growth, at least in part, by increasing GA_1 levels.

PsGA3ox1 Is Not an Auxin Early-Response Gene

Northern analysis showed that IAA-induced *PsGA3ox1* up-regulation was inhibited by the protein synthesis inhibitor CHX (Fig. 6, compare lanes 2 and 4). This indicates that *LE*, although rapidly up-regulated by auxin, is not a true auxin early-response gene. Consistent with this observation, there is no evidence that the *PsGA3ox1* promoter contains any of the classical auxin response elements (Guilfoyle et al., 1998). It seems that one or more protein factors need to be synthesized for IAA to up-regulate *PsGA3ox1* transcription. It will be interesting to see whether any genes already identified as auxin early-response genes in pea may play a role in mediating the IAA regulation of GA biosynthesis. The products of these genes are potential candidates for protein factors involved in the perception of increased IAA levels or subsequent signal transduction.

A Quantitative Relationship Exists between IAA and GA_1 Levels

IAA appears to be a critical determinant of GA_1 levels, and therefore elongation, over a physiologically realistic range of IAA levels. The point for intact plants was on the dose response curve in Figure 7, just before it began to level off with increasing IAA content. This indicates that, at least in this experiment, the level of IAA in intact plants was almost sufficient for maximum GA_1 production. The data also indicate that as the IAA content drops below the intact value, there is a substantial and progressive decrease in the GA_1-synthesizing capacity of the tissue. Extrapolation of the dose response curve shows that it intersects the axes at close to the zero point, suggesting that if there is no auxin present, there is little or no production of GA_1.

It has been suggested that the large span of hormone levels (several orders of magnitude) in many dose response curves indicates systems that are unresponsive to changes in hormones level, providing little evidence for the view that changes in hormone level regulate plant processes (Trewavas, 1983, 1991). It is interesting, therefore, that the total "dose" variation in Figure 7 is only between 1 and 2 orders of magnitude. One reason for this relatively small variation may be the plotting of actual IAA level in the internodes rather than the level applied.

Other Auxins Promote GA_1 Biosynthesis

IAA is not the only auxin that can induce the transcription of auxin-responsive genes. Two IAA-inducible genes isolated from pea epicotyl tissue, *pIAA4/5* and *pIAA6*, were transcribed when tissue was incubated with 2,4-dichlorophenoxyacetic acid (2,4-D) or naphthalene-1-acetic acid (NAA; Theologis et al., 1985). Abel et al. (1995) found that both NAA and 2,4-D were also highly effective in inducing the transcription of *IAA1*, an auxin-inducible gene from Arabidopsis. An increase in *PsGA20ox1* transcript levels has also been observed in deseeded pea pericarps, when treated with 4-chloroindole-3-acetic acid (van Huizen et al., 1997). The current study shows that the promotion of GA_{20} 3-oxidation and inhibition of GA_{20} 2-oxidation are not specific to IAA (Table I). All auxins tested produced a discernible GA_8 peak, although IBA, an auxin present in root and epicotyl tissue (Schneider et al., 1985), was the weakest at promoting GA_1 (and therefore GA_8) biosynthesis. It seems the auxin/GA interaction first demonstrated by van Huizen et al. (1997) and Ross et al. (2000) could be a widespread and important interaction in plant growth regulation.

Evidence That Decapitation Disrupts the Homeostatic Regulation of *PsGA20ox1* Transcription

In GA_1-deficient mutants such as *na*, transcript levels of *PsGA20ox1* are significantly higher than in isogenic WT controls and are reduced by the application of bioactive GAs (Martin et al., 1996; Ross et al., 1999). This is because of "feedback regulation" (Hedden and Croker, 1992), and there is evidence that feedback of *PsGA20ox1* can occur in internodes (Elliott et al., 2001). However, in the current study, the reduction of GA_1 biosynthesis by decapitation did not lead to an up-regulation in the level of *PsGA20ox1* transcript, even though the tissue harvested for RNA extraction had been decapitated for a minimum of 36 h (Fig. 2D). Furthermore, IAA application did not affect *PsGA20ox1* mRNA levels, despite its expected elevation of GA_1 content. A possible explanation for these observations is that the apical bud is necessary for feedback regulation of the *PsGA20ox1* gene in internodes. This would explain why feedback regulation does not occur in decapitated peas, yet does occur in intact GA_1-deficient mutants with a similar reduction in endogenous GA_1 levels. An alternative explanation is that *PsGA20ox1* is actually an IAA-regulated gene, with the down-regulation of mRNA level in (IAA-deficient) decapitated plants canceled out by an up-regulation attributable to GA_1 deficiency.

The level of *PsGA20ox1* transcript in elongating internodes of intact plants was low (Fig. 2D, lane 11). Interestingly, northern analysis of *PsGA20ox1* by Elliott et al. (2001) also showed low levels of this transcript in internode tissue, yet leaflet tissue contained comparatively high-*PsGA20ox1* transcript levels. These findings support a model suggested by Garcia-Martinez et al. (1997) where leaves constitute

a reservoir of GA_{20} to be metabolized to the active GA_1 in internode tissue, in response to stimulating signals (e.g. IAA).

Summary of the Auxin-GA Interaction

The functions of many auxin-regulated genes remain unknown (Abel and Theologis, 1996; Guilfoyle, 1998). It is significant, therefore, that the auxin-regulated GA genes discussed here have well-defined functions, characterized by functional assays of the isolated genes and by the effects of mutations. *PsGA3ox1* (Mendel's *LE*) is a structural gene encoding the enzyme for the conversion of GA_{20} to GA_1 (Lester et al., 1997). The critical importance of this step for growth is indicated by the dramatic dwarfing effect of Mendel's famous *le-1* mutation. Our present results establish that *PsGA3ox1* is rapidly (within hours) up-regulated by auxin (Fig. 2). Nevertheless, *PsGA3ox1* does not appear to be a primary auxin response gene (Fig. 6), but rather a secondary or "late" gene that responds in turn to the initial auxin up-regulation of a primary gene or genes.

It appears that IAA not only promotes GA_1 biosynthesis, but also inhibits GA_1 deactivation (Figs. 3 and 5). Possibly because of this "double-barreled" effect, even moderate changes in IAA supply can lead to physiologically significant changes in GA_1 content (Fig. 7). The effect of IAA on deactivation appears to result from a down-regulation of *PsGA2ox1* expression (Fig. 2). In decapitated plants, *PsGA2ox1* is probably the main gene for GA_1 deactivation, because the *sln* mutation appeared to inhibit the step GA_1 to GA_8 (Fig. 4). We suggest that an important function of IAA in the intact plant is to suppress the expression of *PsGA2ox1*.

We also show that elongating pea stems, in which GA_1 is thought to act, are themselves capable of GA_1 biosynthesis (Fig. 5). There is, in fact, no evidence that endogenous GA_1 is a mobile hormone in pea shoots (Reid et al., 1983). Instead, we suggest that the role of mobile hormone is performed by IAA, with GA_1 acting as an actual effector of elongation in the internodes (Ross and O'Neill, 2001). By allowing us to assign these roles to the two hormones, our recent findings have shed new light on the auxin-GA interaction.

MATERIALS AND METHODS

Plant Material

For the majority of experiments, the line used was the tall (WT) line 205+. Where specified line 250− (*sln*) was used. Line 250− is near-isogenic with the line 250+ (*SLN*) as described by Lester et al. (1999). Plants were grown, two per pot, in a heated greenhouse as described previously (Beveridge and Murfet, 1996). The photoperiod was 18 h, provided by extending the natural photoperiod at its beginning and end with a mixture of white fluorescent (40-W) and incandescent (100-W) lights (intensity 25 μmol m^{-2}s^{-1} at the soil surface). All node counts began from the cotyledons as zero. Internode 6-7 was the internode between nodes 6 and 7.

Treatments and Harvest

For IAA time-course experiments, we chose plants with the internode between node 7 and 8 (internode 7-8), approximately 20–40 mm long. These plants were either decapitated approximately 15 mm above node 7 and pure lanolin (Biotech Pharmaceuticals P/L, Carole Park, Queensland, Australia) was applied to the stump, or they were left intact. After 38 h, one-half of the decapitated plants were retreated with lanolin only, whereas the other half received IAA (Sigma-Aldrich, St. Louis) in lanolin (3,000 μg g^{-1}; approximately 15 mg of lanolin per plant). IAA/lanolin or pure lanolin was reapplied 4 h after the first IAA application, with the previously applied lanolin removed before reapplication to ensure a continuous supply of IAA in the decapitated internode. Internode 7-8 tissue (seven plants per time point) was harvested for RNA extraction (after removal of the applied lanolin) at 0, 2, 4, 6, and 8 h after the first IAA application to examine the short-term effect of IAA on the transcript level of key GA biosynthesis genes. Harvested material was immersed in liquid nitrogen and immediately stored at −70°C until RNA extraction took place.

To investigate the effect of IAA on [^{14}C]GA_1 metabolism, 205+ plants with nine fully expanded leaves were decapitated 15 mm above node 9. IAA (3,000 μg g^{-1}) was applied to one-half the plants and reapplied after a further 8, 20, and 28 h. Twenty-two hours after decapitation, all plants were treated with [^{14}C]GA_1 (34 mCi mmol^{-1}) at a rate of 80,000 dpm plant^{-1} on leaf 8 in 10 μL of ethanol (direct application to the internode was not successful). Five hours after the application of [^{14}C]GA_1, the treated leaf was excised, and internode 8-9 was harvested 10 h after [^{14}C]GA_1 application. Two replicate batches (10 or 11 plants in each) were harvested for each of the control and IAA treatments.

The analysis of [^{14}C]GA_1 metabolism in *SLN/sln* plants was carried out using plants grown for 33 d. At this stage, the plants possessed 10 fully expanded leaves, and internode 10-11 was 10 to 50 mm in length. The apical bud was excised 10 to 20 mm above node 10. Nineteen hours after decapitation, plants were treated with [^{14}C]GA_1 at a rate of 80,000 dpm plant^{-1} on leaf 9 in 10 μL of ethanol. Five hours after application of [^{14}C]GA_1, the treated leaf was excised, and internode 9-10 was harvested 9 h after application. All harvested internodes were immediately immersed in cold 80% (v/v) methanol before extraction and HPLC-radiocounting analysis as methyl esters.

Internode segments were cultured in liquid Murashige and Skoog solution first by cutting 20-mm segments from the uppermost section of 10% to 50% expanded internode 7-8 tissue. For each treatment, eight of these segments were placed in a separate petri dish with 10 mL of sterile Murashige and Skoog solution containing (depending on the treatment) IAA (5 μg mL^{-1}) and/or CHX (14 μg mL^{-1}) added in 100 μL of methanol, or 100 μL of methanol in the case of controls. Other auxins tested were: 2,4-D (PAL Chemicals, London), NAA (Sigma-Aldrich), [^{13}C]IBA (Chemistry Department, University of Tasmania), and 4-chloroindole-3-acetic acid (from Dr. V. Magnus, Ruoer Boskovic Institute, Zagreb, Croatia). On the basis of experience with other [^{13}C]-labeled phytohormones, [^{13}C]IBA was not expected to differ markedly in its biological activity from unlabeled IBA. For metabolism studies, [^{14}C]GA_{20} (55 mCi mmol^{-1}) was added to the incubation medium immediately after the stem segments at a concentration of 0.4 μM. Petri dishes were incubated in a controlled environment room with a temperature of 20°C ± 2°C and a photoperiod of 16 h of white light supplied by white fluorescent tubes (cool-white 5G, 36W/W43, Thorn, Smithfield, Australia). Incubation times ranged from 4 to 24 h, as detailed for each experiment. This technique was based on the work of Smith (1992). After the incubation period, material for GA or IAA analyses was rinsed twice in distilled water to remove residual substrate, then immersed in cold (−20°C) 80% (v/v) methanol, and stored in a freezer at −20°C. Material for northern analysis was immersed in liquid nitrogen and stored at −70°C till RNA extraction took place.

For the IAA dose response study in Figure 7, plants with internode 7-8 10% to 50% expanded were decapitated 10 to 20 mm above node 7 or were left intact. Immediately after decapitation, the stumps were treated with pure lanolin (controls) or lanolin paste of varying IAA concentration, from 0.1 to 3 mg IAA g^{-1} lanolin. Each dose of IAA/lanolin was then reapplied 8, 24, 32, and 40 h after the original application. Intact plants remained untreated. The length of internode 6-7 tissue was measured from all plants (decapitated and intact groups) at 24 and 48 h after the original IAA application. After the 48-h measurement, each treatment group of eight plants was split into two replicate harvests of four plants each. Each harvest

was then immersed in cold (−20°C) 80% (v/v) methanol and stored in a freezer at −20°C for GA and IAA quantification.

Extraction and Analyses

For GA and IAA analyses, tissue was homogenized, hormones were extracted at 4°C for 24 h, and extracts were then filtered (No. 1, Whatman, Clifton, NJ) using a Buchner apparatus. Endogenous GAs and IAA were analyzed by gas chromatography-mass spectrometry-selected ion monitoring (GC-MS-SIM) with internal standards, as described previously (Ross, 1998), but using $^{13}C_6$ IAA (Cambridge Isotope Laboratories, Woburn, MA) as an internal standard for IAA quantification. For $[^{14}C]GA_1$ and $[^{14}C]GA_{20}$ metabolism experiments, a small aliquot of the filtrate was taken for radio-counting (to estimate the total radioactivity), with either one-half or all of the remaining filtrate purified using Sep-Pak Vac RC C18 cartridges (Waters, Milford, MA) and chromatographed as methyl esters as described previously (Ross, 1998).

Tissue for RNA extraction was ground in liquid nitrogen, and total RNA was extracted using either a phenol/SDS method (Ausubel et al., 1994) or an RNeasy Plant Mini Kit (Qiagen Pty Ltd, Clifton Hill, Victoria, Australia) and quantified using either a Smart Spec 3000 (Bio-Rad Laboratories, Hercules, CA) or a Lambda 20 UV/VIS spectrometer (PerkinElmer Life Sciences, Boston). Unless otherwise indicated, 10 μg of total RNA per lane was electrophoresed in a formaldehyde gel, blotted to GenescreenPlus (NEN, Boston), and hybridized overnight in formamide prehybridization solution as detailed by Ausubel et al. (1994). Oligo-probes were labeled with α-^{32}P by random oligonucleotide-primed synthesis using a DecaLabel DNA labeling kit (MBI Fermentas, Progen, Queensland, Australia). Northern blots were first washed in 2× SSC and 0.1% (w/v) SDS at 42°C followed by a second wash of 0.2× SSC and 0.1% (w/v) SDS at 65°C (a second wash of 0.1× SSC and 0.1% [w/v] SDS at 65°C was used for *PsGA20ox1* blots). Blots were exposed to x-ray film (Biomax MS, Eastman-Kodak, Rochester, NY) at −70°C with a Biomax MS intensifying screen.

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